FEDEROFF DECLARATION

Exhibit B

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Versatile adeno-associated virus 2-based vectors for constructing recombinant virions

(Recombinant DNA; DNA rescue and replication; DNA packaging; parvovirus; gene therapy)

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SUMMARY

We have constructed several plasmid vectors with which a more efficient molecular cloning, followed by rescue, replication, and packaging of DNA fragments, can be achieved. The availability of these vectors should facilitate construction of a variety of recombinant adeno-associated virus 2 (AAV)-based virions containing therapeutic genes for potential use in human gene therapy.

INTRODUCTION

It is becoming increasingly clear that in addition to the relatively high viral titers, higher infectivity, and stability of human parvoviruses (Berns and Bohenzky, 1987; Berns, 1990), parvovirus-based vectors may be a potentially useful alternative to the more commonly used retroviral vectors for gene therapy in humans (Hermonat and Muzyczka, 1984; McLaughlin et al., 1988; Samulski et al., 1989; 1991; Srivastava et al., 1989; 1990; Kotin et al., 1990; Nahreini et al., 1992; P.N., S.Z.Z. and A.S., submitted). For example, a non-pathogenic human parvovirus, the adeno-associated virus 2 (AAV), is so far not known to be associated with any human disease, but has recently been documented to integrate into the human chromosome site-specifically (Kotin et al., 1990; Samulski et al., 1991; Nahreini et al., 1992; P.N., S.Z.Z. and A.S., submitted). In addition, a pathogenic human parvovirus, designated B19 (Cossart et al., 1975), has been shown to have a remarkable tissue-tropism for the erythroid lineage in human hematopoietic cells (Ozawa et al., 1986). We have described the construction of a hybrid AAV-B19 genome, and speculated on its potential utility as a vector for gene transfer in human bone marrow cells (Srivastava et al., 1989).

However, the currently available preferred method of constructing recombinant AAV genomes utilizes a plasmid, designated p*sub*201 (Samulski et al., 1987), in which the two engineered *Xba*I sites are used to remove the AAV coding region, and a gene of interest is inserted between the two AAV inverted terminal repeats (ITRs). The inserted gene can be subsequently rescued and packaged into mature AAV virions following cotransfection with a helper plasmid, pAAV/Ad (Samulski et al., 1989) in adenovirus (Ad)-infected human cells (Srivastava et al., 1989; 1990). Since the AAV-ITRs are approximately 70% GC-rich, and are also palindromic (Lusby et al., 1980; Srivastava et al., 1983), as free-ends they can rapidly form hairpin structures that are unstable in bacterial cells (Samulski et al., 1983). As a consequence, the cloning efficiency using the *Xba*I-cleaved

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Abbreviations: AAV, Ad-associated virus 2; Ad, adenovirus; bp, base pair(s); Ap, ampicillin; Cap, capsid proteins; G418, Geneticin (Gt); ITR, inverted terminal repeat; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); *o*, operator; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; *ori*, origin of DNA replication; Rep, replication proteins; ^{R}, resistance/resistant; Tc, tetracycline; TK, thymidine kinase.

p*sub*201 DNA can be significantly reduced. In this report, we describe the construction of several plasmid vectors which can be efficiently used to construct a variety of recombinant AAV genomes containing the gene(s) of interest.

EXPERIMENTAL AND DISCUSSION

(a) Construction of recombinant plasmids pWP-7A and pWP-19

The general overall strategy used to construct the prototype plasmid vectors, designated pWP-7A and pWP-19, respectively, is depicted in Fig. 1.

Whereas plasmid pWP-7A is useful for cloning genes up to 2.5 kb in size at the unique *Eco*RI site, plasmid pWP-19 offers a built-in *neo*^{R} marker gene as well as a number of unique cloning sites such as *Sac*I, *Kpn*I and *Bam*HI, and genes up to 2.8 kb in size can be inserted between the two AAV-ITRs.

(b) Construction of a novel recombinant plasmid pWN-1

The strategy to construct the recombinant plasmid pWN-1 is shown in Fig. 2. This plasmid can be cleaved with *Xba*I to generate the Tc^R fragment flanked by the two AAV-ITRs in an opposite orientation. Since the Tc^R gene fragment lacks the *ori* sequence, any plasmid DNA containing the *ori* sequence can be ligated to this frag-

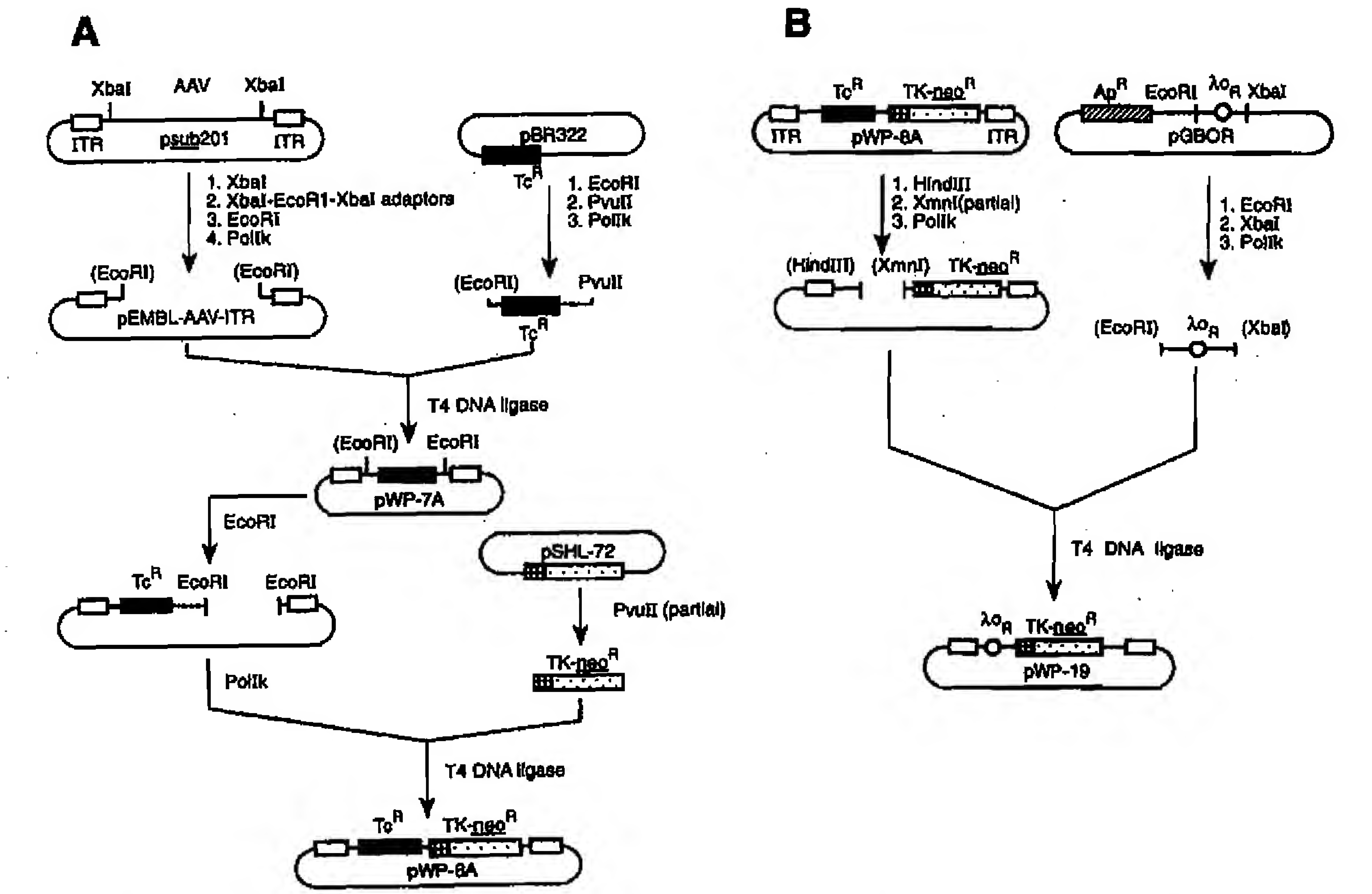

Fig. 1. The strategy for the construction of recombinant plasmids pWP-7A and pWP-8A (A), and pWP-19 (B). The *Xba*I sites in plasmid p*sub*201 were converted to *Eco*RI sites by ligating synthetic *Xba*I-*Eco*RI-*Xba*I adaptors as previously described (Srivastava et al., 1989). The AAV coding region was removed following digestion with *Eco*RI, and the vector DNA containing the two AAV-ITRs was isolated from preparative agarose gels (Seth, 1984), and treated with PolIk to generate blunt-ends. Similarly, pBR322 DNA was cleaved with *Eco*RI + *Pvu*II and a 2066-bp fragment containing the entire Tc^R was also blunt-ended using PolIk. These two fragments were ligated and used to transform competent *E. coli* HB101 cells by the standard methods described in Sambrook et al. (1989) to generate a plasmid, designated pWP-7A. Since blunt-end ligation of DNA fragments containing repaired *Eco*RI and *Pvu*II ends regenerates an *Eco*RI site, plasmid pWP-7A can be cleaved with *Eco*RI downstream from the Tc^R gene for cloning a gene of interest. We chose a gene for resistance to neomycin (*neo*^{R}), expression of which can be easily detected in human cells following selection with the drug G418. The *neo*^{R} gene under the control of the herpesvirus thymidine kinase (TK) promoter was isolated from plasmid pSHL-172 (Tratschin et al., 1985) by partial digestion with *Pvu*II, and blunt-end ligated with PolIk-treated pWP-7A DNA. The resulting recombinant plasmid, designated pWP-8A, is shown in panel A. Plasmid pWP-19 was constructed as follows. Plasmid pWP-8A was linearized with *Hin*dIII, which cleaves at the 5′ end of the Tc^R gene, and partially digested with *Xmn*I to remove the Tc^R gene. A plasmid pGBOR which contains an Ap^R gene and the bacteriophage λ operator ($o_R1/o_R2 = \lambda o$) sequences (Samulski et al., 1991), was cleaved with *Eco*RI + *Xba*I and the fragment containing the λo sequence was blunt-end ligated with PolIk-treated pWP-8A DNA described above. The resulting recombinant plasmid pWP-19 is shown in panel B.

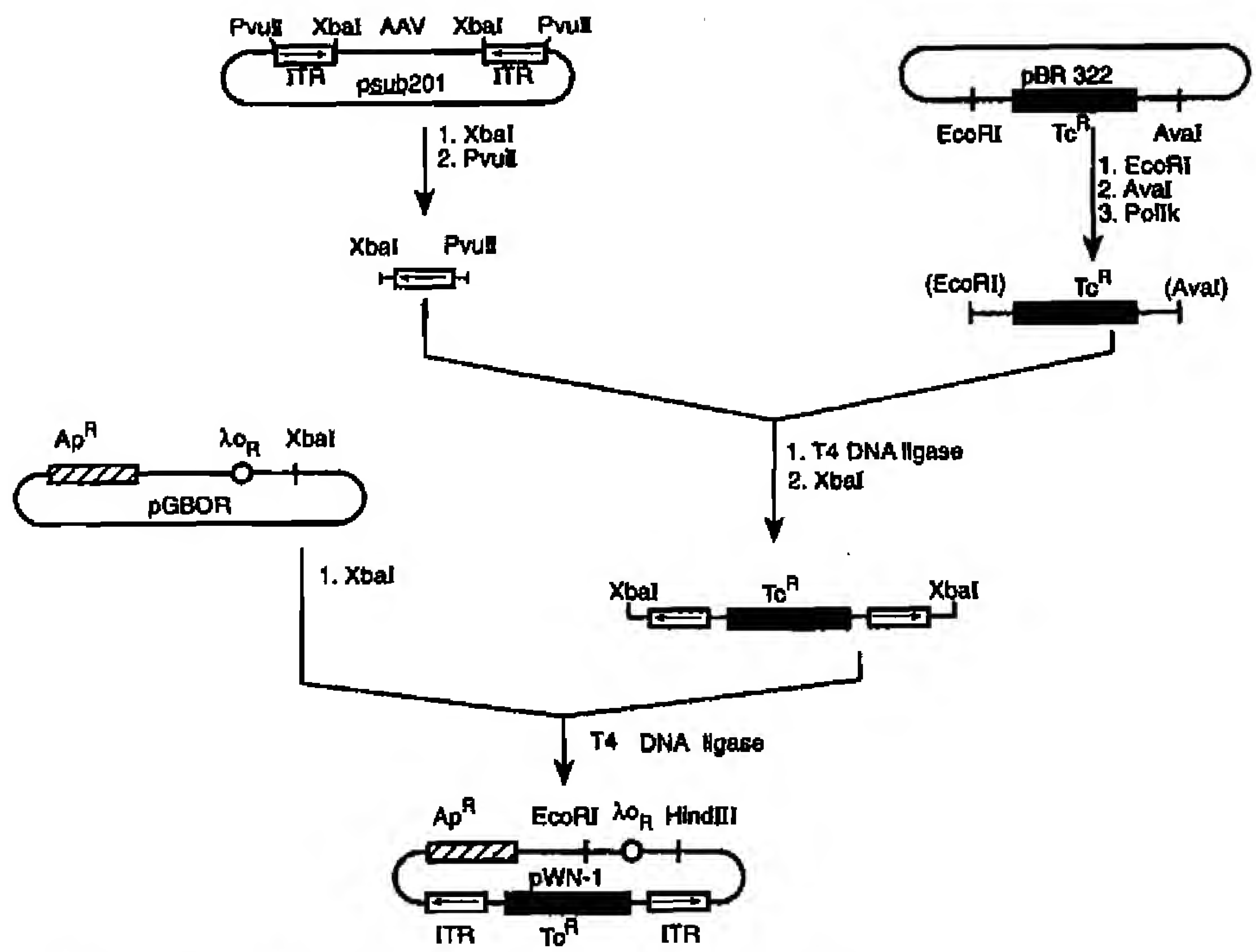

Fig. 2. The strategy for the construction of recombinant plasmid pWN-1. Briefly, p*sub*201 plasmid DNA was digested to completion with *Xba*I + *Pvu*II, and a 191-bp *Xba*I-*Pvu*II fragment containing the entire AAV-ITR sequence was isolated as described in the legend to Fig. 1. Similarly, pBR322 plasmid DNA was cleaved with *Eco*RI + *Ava*I to isolate a 1425-bp fragment that contains the Tc^R gene but lacks the *ori* sequence. This fragment was treated with PolIk to generate blunt ends. Blunt-ended *Eco*RI-*Ava*I fragment was mixed with a large excess of the *Xba*I-*Pvu*II fragment containing the AAV-ITR, blunt-end ligated using T4 DNA ligase, and then digested exhaustively with *Xba*I. This resulted in the production of the Tc^R gene flanked by a single AAV-ITR at each end but in the opposite orientation (see small arrows in boxed ITRs). This fragment was subsequently ligated at the unique *Xba*I site in plasmid pGBOR described in the legend to Fig. 1, and Tc^R was used to select for the recombinant plasmid pWN-1.

ment and recombinants obtained following selection with Tc.

(c) Rescue and replication of the *neo*^{R} gene from recombinant AAV-based plasmids

We next wished to examine whether DNA sequences flanked by the two AAV-ITRs could be rescued from these recombinant plasmids following transfection in human cells in the presence of the AAV and Ad proteins as a prelude to successful packaging of these genes into mature AAV virions (Samulski et al., 1989; Srivastava et al., 1989; 1990). The insert size between the two AAV-ITRs in plasmid pWP-8A is similar to that of the wt AAV genome, and the AAV-*rep* gene from the parent plasmid p*sub*201, and the λ o_R1/o_R2 sequences from plasmid pGBOR were isolated and inserted in plasmid pWP-19 by the strategy shown in Fig. 3. Two recombinant plasmids, pWP-21 and pWP-22, were generated which contain the AAV-*rep* gene in different orientations with respect to the *neo*^{R} gene. These plasmids are depicted in Fig. 3A. Similarly, the insert size between the two AAV-ITRs in plasmid pWN-1 was increased by inserting the *neo*^{R} gene either at the *Nde*I site or at the *Pst*I site to generate two recombinant plasmids, pWP-16 and pWP-17, respectively, which are shown in Fig. 3B. Plasmids pWP-8A, pWP-21, pWP-22, pWP-16 and pWP-17 were either transfected alone, or co-transfected with the AAV helper plasmid (pAAV/Ad) separately, in Ad-infected human KB cells (Samulski et al., 1989; Srivastava et al., 1989; 1990). Low-M_r DNA samples were isolated, digested with *Dpn*I, and analyzed on Southern blots (Southern, 1975) using a *neo*-specific DNA probe as previously described (Samulski et al., 1989; Nahreini et al., 1992). The results of these experiments are presented in Fig. 4. It is interesting to note that whereas no rescue/replication of the recombinant *neo*^{R} gene from plasmid pWP-8A occurred in the absence of the pAAV/Ad helper plasmid (lane 1), successful rescue and replication indeed occurred when the AAV-Rep proteins were supplied *in trans* (lane 2), as detected by the presence of the characteristic monomeric and dimeric replicative intermediates of the recombinant AAV genome. Similarly, rescue and replication occurred from plasmids pWP-21 (lanes 3 and 4), and pWP-22 (lanes 5 and 6) even in the absence of the helper plasmid because these plasmids contain the AAV-*rep* gene in cis. Rescue and replication from plasmids pWP-16 and pWP-17 also occurred, but only in the presence of the AAV helper plasmid (lanes 8 and 10).

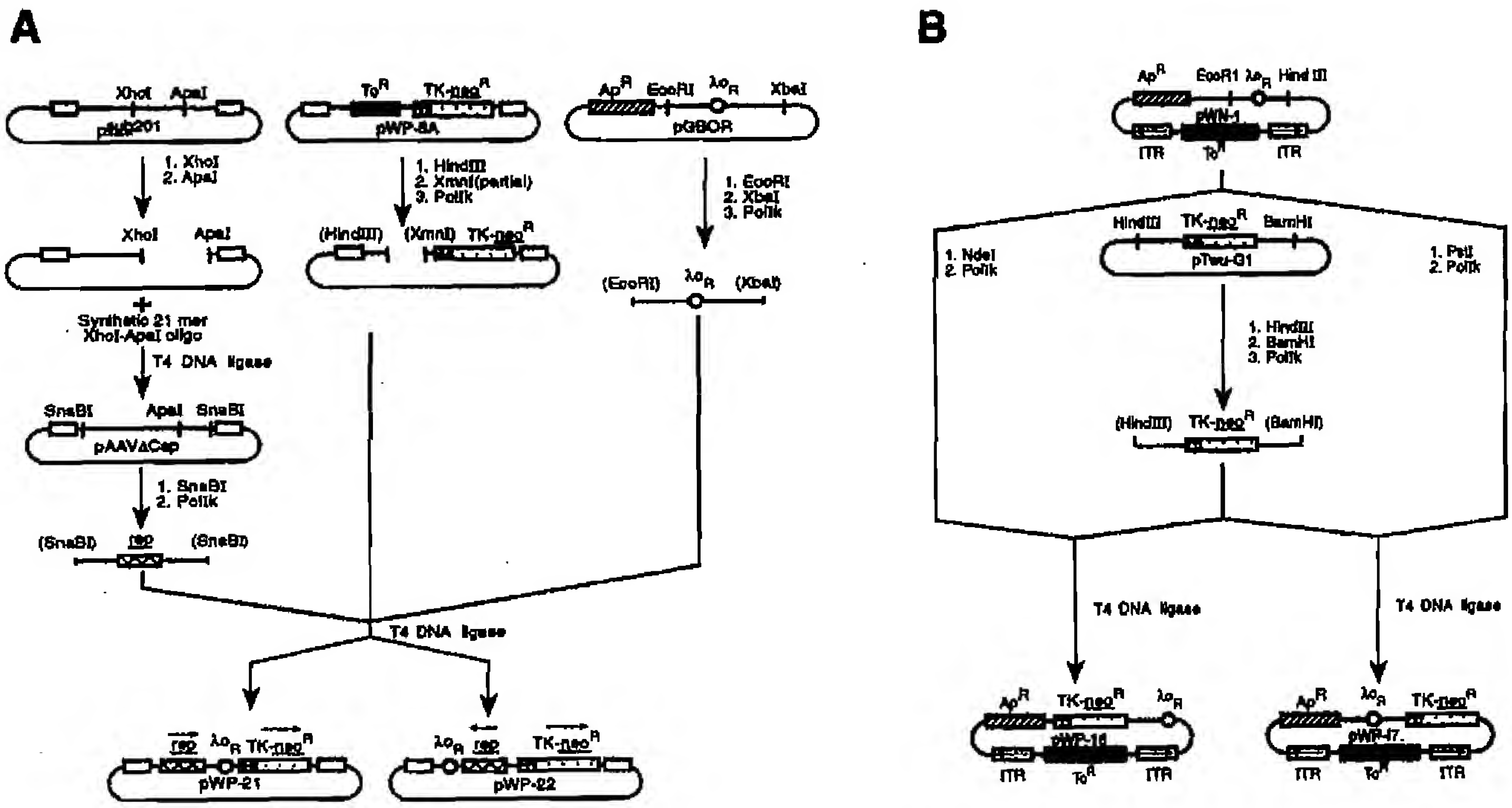

Fig. 3. The strategy for the construction of recombinant plasmids pWP-21 and pWP-22 (A), and pWP-16 and pWP-17 (B). Briefly, plasmid p*sub*201 was cleaved with *Xho*I + *Apa*I to remove the AAV-*cap* gene and the ends were religated using a synthetic oligo (5'–TCGAGGACACTCTCTCTGAAG) containing the *Xho*I and *Apa*I cohesive ends. The AAV-*rep* gene sequence was isolated from the resulting plasmid, designated pAAVΔCap, following digestion with *Sna*BI. Similarly, plasmid pWP-8A was cleaved with *Hin*dIII + *Xmn*I (partial), and plasmid pGBOR was cleaved with *Eco*RI + *Xba*I, to obtain the pWP-8A vector lacking the Tc^R gene, and the λ o_R1/o_R2 sequences, respectively. All DNA fragments were treated with PolIk and used in a three-way ligation reaction to generate two recombinant plasmids, designated pWP-21 and pWP-22, respectively. Recombinant plasmids pWP-16 and pWP-17 were constructed as follows. Plasmid pWN-1 was cleaved with either *Nde*I or *Pst*I, treated with PolIk, and ligated with PolIk-treated TK-*neo*^{R} DNA fragment isolated from plasmid pTwuG1 following digestion with *Hin*dIII + *Bam*HI.

Following rescue and replication, the *neo*^{R} gene could also be packaged into mature AAV progeny virions in presence of the AAV-Cap proteins. The recombinant AAV progeny virions were biologically active and infectious. For example, recombinant AAV-*neo* virions were used to transduce and stably integrate the *neo*^{R} gene in a variety of diploid and polyploid human cells. The transduced *neo*^{R} gene was biologically active, as determined by gene expression analyses on Northern blots, as well as by ready isolation of clonal populations of human cells that were resistant to G418 (Nahreini et al., 1992; P.N., S.Z.Z. and A.S., submitted).

(d) Conclusions

The plasmid vectors pWP-7A and pWP-19 are useful for constructing recombinant AAV genomes because direct insertion of a gene of interest is possible in both, and the presence of the built-in *neo*^{R} gene in pWP-19 provides a strong selectable marker in human cells. The plasmid vector pWN-1 is particularly useful, in comparison to p*sub*201, because it offers several advantages. In bacterial cells, these include: (*1*) The availability of a variety of unique cloning sites (*Bam*HI, *Sac*I, *Kpn*I, *Xba*I, and *Pst*I), including the *Nde*I site; (*2*) Stable AAV-ITRs because they are well-separated from the cloning sites (for example, the *Eco*RI site is located 111 bp and 2711 bp, respectively, away from the two AAV-ITRs); and (*3*) The use of Tc^R as well as Ap^R as selectable markers. In mammalian cells, following transfection in the presence of the AAV helper plasmid and Ad, the gene of interest, which is now flanked by the two AAV-ITRs in their proper orientation (see Fig. 2), can be efficiently rescued from the Tc^R gene followed by DNA replication and packaging in the AAV progeny virions as described above. Furthermore, the presence of the λ o_R1/o_R2 (λ*o*) sequence within the transduced chimeric genes may also permit retrieval of the target site for integration in human cells (Samulski et al., 1991). We have also inserted a number of biologically relevant genes in these vectors and constructed recombinant AAV virions to test their therapeutic potential following AAV-mediated gene transfer (S.Z.Z., P.N. and A.S.; F. Luo, S.Z.Z. and A. S., unpublished results).

One limitation of these vectors in general is the maximum size of the DNA fragment, which is approx. 2.5 kb, that can be inserted in these vectors and successfully packaged in mature AAV progeny virions. However, it is

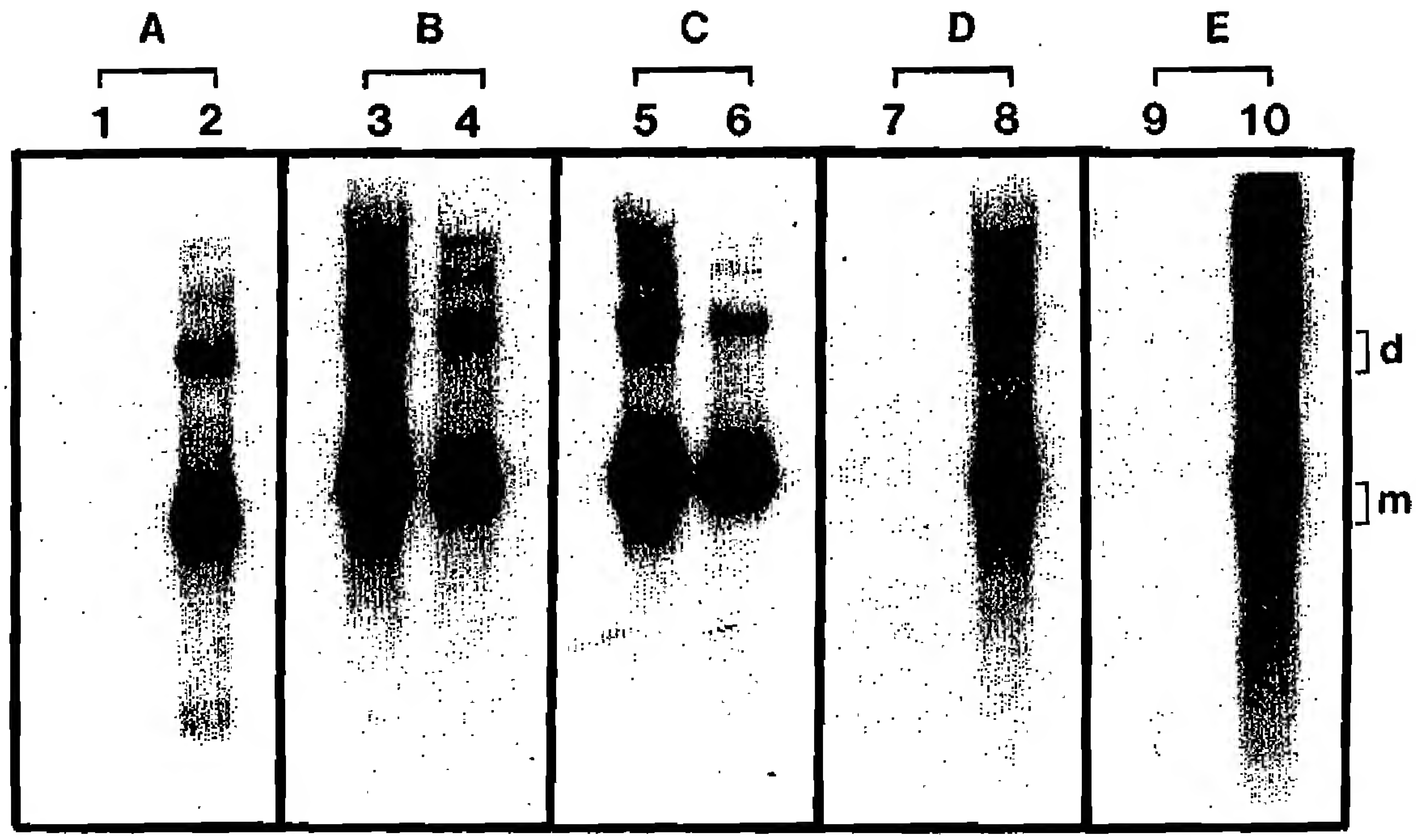

Fig. 4. Southern blot analysis of rescue and replication of the recombinant *neo*^{R} gene in human cells. Panel A: rescue from plasmid pWP-8A; panel B: rescue from plasmid pWP-21; panel C: rescue from plasmid pWP-22; panel D: rescue from plasmid pWP-16; panel E: rescue from plasmid pWP-17. Recombinant plasmids were transfected separately in Ad-infected human KB cells (lanes 1, 3, 5, 7, 9), or co-transfected with the pAAV/Ad helper plasmid (lanes 2, 4, 6, 8, 10). Low-M_r DNA samples were isolated by the method described by Hirt (1967) 72 h post-infection/transfection, digested with *Dpn*I, electrophoresed on 1% agarose horizontal slab gels, and analyzed by Southern blots using a *neo*-specific DNA probe as previously described (Nahreini et al., 1992). m and d denote the monomeric and dimeric forms, respectively, of the recombinant AAV DNA replicative intermediates.

possible to delete the entire coding region of the Ap^R gene from pWN-1 in order to accommodate larger pieces of DNA, up to approx. 4.0 kb in size. These vectors should, nonetheless, prove useful for cloning a variety of human cDNA molecules.

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